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Advanced glycation end products inhibit Na^+ K^+ ATPase in proximal tubule epithelial cells: Role of cytosolic phospholipase $A_2\alpha$ and phosphatidylinositol 4-phosphate 5-kinase γ

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ABSTRACT

Chronic hyperglycaemia during diabetes leads to non-enzymatic glycation of proteins to form advanced glycation end products (AGEs) that contribute to nephropathy. In diabetes, renal Na^+ K^+ ATPase (NKA) activity is downregulated and phosphoinositide metabolism is upregulated. We examined the effects of AGEs on NKA activity in porcine LLC-PK1 and human HK2 proximal tubule epithelial cells. AGE-BSA increased cellular phosphoinositol 4,5 bisphosphate (PIP2) production as determined by immunofluorescence microscopy and thin layer chromatography. AGE-BSA (40 μM) induced ^{3}H-arachidonic acid release and reactive oxygen species (ROS) production via cytosolic phospholipase A_2 ($cPLA_2$) activation. Within minutes, AGE-BSA significantly inhibited NKA surface expression and activity in a dose- and time-dependent manner as determined by immunofluorescence staining and [$^{86}Rb^+$] uptake, respectively, suggesting AGEs inhibit NKA by stimulating its endocytosis. The AGE-BSA-induced decrease in cell surface NKA was reversed by a $cPLA_2\alpha$ inhibitor, neomycin, a PIP2 inhibitor, and PP2, a Src inhibitor. AGE-BSA increased binding of NKA to the α-adaptin but not β2- or μ2-adaptin subunits of the AP-2 clathrin pit adaptor complex. Transfection of HK2 cells with PIP5Kγ siRNA prevented AGE-BSA inhibition of NKA activity. AGEs may stimulate PIP5Kγ to increase PIP2 production, which may enhance AP-2 localisation to clathrin pits, increase clathrin pit formation, enhance NKA cargo recognition by AP-2 and/or stimulate $cPLA_2\alpha$ activity. These results suggest AGEs modulate arachidonic acid and phosphoinositide metabolism to inhibit NKA via clathrin-mediated endocytosis. Elucidation of new intracellular AGE signaling pathways may lead to improved therapies for diabetic nephropathy.

1. Introduction

Nonenzymatic glycation of proteins, lipids and nucleic acids resulting in the accumulation of advanced glycation end products (AGEs) correlates with the development of chronic diabetic complications such as retinopathy, nephropathy, neuropathy and vasculopathy [1–3]. Injection of AGEs into rats leads to renal changes similar to those of diabetic nephropathy while interfering with AGE formation reduces diabetic complications in various animal models [4–6]. AGE formation and accumulation precedes diabetic renal disease suggesting AGEs have a role in the pathogenesis of diabetic nephropathy [2]. Additionally, serum and tissue AGE levels are increased in diabetic nephropathy while AGEs cause renal cell dysfunction *in vitro* [7–9]. However, the precise cellular mechanisms whereby AGEs promote the development of diabetic complications remain unknown.

AGEs regulate arachidonic acid metabolism. In neutrophils, AGEs stimulate an enhanced respiratory burst by activating cytosolic phospholipase A_2 ($cPLA_2$) to catalyse the hydrolysis of membrane phospholipids to selectively release free arachidonic acid [10]. Arachidonic acid is a substrate for production of epoxyeicosatrienoic acids by cytochrome P450 enzymes, prostaglandins by cyclooxygenase and leukotrienes by 5-lipooxygenase, all of which all then generate reactive oxygen species (ROS) [11].

Phosphatidylinositol 4,5 bisphosphate (PIP2) is a major membrane phospholipid that affects cellular processes such as actin cytoskeleton modulation, membrane trafficking and ion channel activity [12]. PIP2 is generated mainly via phosphorylation of phosphatidylinositol-4-phosphate (PI4P) by phosphatidylinositol 4-phosphate 5-kinase (PIP5K). In diabetic rats, [^{32}P] orthophosphate incorporation into PIP2 is preferentially increased in peripheral nerves [13] while platelet uptake of 2-[^{3}H] myo-inositol, a measure of phosphoinositide

Abbreviations: BSA, bovine serum albumin; LLC-PK1, porcine kidney proximal tubule epithelial cells; HK2, human kidney proximal tubule epithelial cells; SFM, serum free medium; GM, growth medium; NKA, sodium potassium ATPase; PIP2, phosphatidylinositol 4,5 bisphosphate; ROS, reactive oxygen species; $cPLA_2\alpha$, cytosolic phospholipase A_2 alpha; AGEs, advanced glycation end products; PIP5Kγ, phosphatidylinositol 4-phosphate 5-kinase γ; AP-2, adaptor protein complex 2

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synthesis, is also increased [14]. Apart from these examples, however, there is a paucity of studies relating to phosphoinositide metabolism during diabetes.

Na^+ K^+ ATPase (NKA) is a basolateral membrane protein pump responsible for maintaining intracellular sodium and potassium balance that, in turn, regulates many other ion and solute transporters [15]. NKA is regulated by many factors including hormones, neuropeptides and glucose. In cultured endothelial and smooth muscle cells, glucose-induced inhibition of NKA activity involves sequential activation of protein kinase C and $cPLA_2$ [16]. NKA is regulated acutely by its internalisation via clathrin-mediated endocytosis, whereas transcription and translation are involved in its long-term regulation.

NKA activity is reduced within erythrocytes of patients with type 1 and insulin-requiring type 2 diabetes and may be related to C-peptide levels [17–23]. A few studies have also suggested a relationship between reduced NKA activity and AGEs. Glycated proteins inhibit NKA activity in normal platelets [24]. In diabetic rats, aminoguanidine and OPB-9195, potent anti-glycation agents, inhibit the loss of sciatic nerve NKA activity [25,26]. However, the mechanisms involved in AGE inhibition of NKA activity have not been elucidated.

We examined the effects of AGEs on NKA activity in two renal proximal tubule epithelial cell lines. We found that AGEs activated $cPLA_2\alpha$ and PIP5Kγ to inhibit NKA surface expression and activity. AGEs stimulated clathrin-mediated endocytosis of NKA by a mechanism that involved enhanced binding of PIP5Kγ to the clathrin adaptor protein complex AP-2. Our studies provide the first *in vitro* evidence of a role for $cPLA_2\alpha$ and phosphoinositide metabolism in AGE-induced inhibition of NKA.

2. Materials and methods

2.1. Materials

Tissue culture plastics and reagents were purchased from Nunc (Roskilde, Denmark) and Trace Biosciences (Melbourne, Australia), respectively. All other laboratory reagents were purchased from Sigma Corporation (St Louis, MO) unless otherwise specified.

2.2. Inhibitors

Inhibitors used in these studies and their final concentrations are: 10 μM quinacrine dihydrochloride for PLA_2, 1 μM AACOCF3 (Calbiochem) for $cPLA_2$, 60 nM $cPLA_2\alpha$ inhibitor (Calbiochem), 1 μM indomethacin (Calbiochem) for cox1/2, 10 nM sc-560 (Calbiochem) for cox-1, 32 μM ns-398 (Calbiochem) for cox-2, 3.5 mM neomycin (Calbiochem) for PIP2, 10 μM proadifen hydrochloride and 8.9 nM HET0016 (Cayman Chemical, Ann Arbor, MI) for cytochrome P450, 2.8 μM nordihydroguaiaretic acid (NDGA) for pan lipoxygenases, 2.5 μM haloenol lactone (E)-6-(bromomethylene)-3-(1-naphtalenyl)-2H-tetrahydropyran-2-one (HELSS; Calbiochem) for Ca^{2+}-independent PLA_2, 1 μM manoalide for secretory PLA_2, 100 nM PP2 or PP3 (Calbiochem) for Src and its negative control, respectively. Since some inhibitors were dissolved in DMSO or ethanol, we tested the effects of these solvents in all assays and found that they had no effect (results not shown).

2.3. AGE preparation

AGE-BSA was prepared as described previously with modifications [27]. Briefly, BSA (10 mg/ml) or RNase (10 mg/ml) was incubated with D-glucose (90 g/l) in 0.4 M phosphate buffer at 37 °C for 12 or 6 weeks, respectively. Preparations were lyophilised, resuspended in water and dialysed against phosphate buffered saline (PBS) to remove free glucose. Control BSA or RNase was prepared by identical incubation without glucose. Glycation was assessed by characteristic fluorescence (excitation 370 nm, emission 440 nm) with a 7-28-fold increase in fluorescence of AGE-BSA compared to BSA.

Methylglyoxal-BSA (MGO-BSA) was prepared as described [28]. Briefly, 0.22 g BSA was incubated with or without 0.54 ml of 40 (w/v)% methylglyoxal in 30 ml 0.4 M phosphate buffer for 24 h and then dialysed against 30 mM ammonium bicarbonate pH 7.8 to remove free methylglyoxal.

2.4. Cell culture

LLC-PK1 porcine and HK2 human kidney proximal tubule epithelial cell lines were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD) [29,30]. Cells were cultured in a 5% CO_2 incubator in growth medium (GM) consisting of Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose, 2 mM L-glutamine, 5000 IU/l penicillin, 5 mg/l streptomycin, 125 U/l Fungizone, 2.2 g/l sodium bicarbonate supplemented with 10% fetal calf serum (FCS). For experiments under serum-free conditions, cells were cultured in serum-free medium (SFM) consisting of DMEM with antibiotics and glutamine supplemented with 0.5 g/l BSA.

2.5. Immunofluorescence

LLC-PK1 cells were grown on glass coverslips. After incubation in SFM overnight, cells were treated with 5–40 μM BSA or AGE-BSA for up to 1 h in time/dose experiments. Cells were also pre-treated with 10 μM quinacrine, 1 μM AACOCF3, 1 μM indomethacin, 60 nM $cPLA_2\alpha$ inhibitor or 3.5 mM neomycin for 15 min and then 20 μM BSA or AGE-BSA for 30 min. LLC-PK1 cells were incubated with 20 μg/ml PGE_2 or 30 nM arachidonic acid. LLC-PK1 cells were also treated with other AGEs to demonstrate specificity: 40 μM human glycated albumin (US Biological, MA), 5–40 μM AGE-RNase, 5–40 μM MGO-BSA or their respective non-glycated controls.

After fixation in 3.7% paraformaldehyde/PBS for 10 min, cells were incubated in 0.1% Triton X-100/PBS for 5 min, blocked with 1% BSA/PBS for 15 min and stained with a 1/50 dilution in 1% BSA/PBS of anti-PIP2 antiserum (Echelon Biosciences, Salt Lake City, UT) for 1 h at room temperature or anti-NKAα subunit (α6F, Developmental Studies Hybridoma Bank, University of Iowa, IA or Sigma) at a dilution of 1/75 overnight at 4 °C. Staining with anti-mouse or anti-rabbit rhodamine- or fluoroscein-labelled secondary antibodies (Invitrogen) was performed for 1 h at a 1/1000 dilution. After washing in PBS and then water, slides were mounted in Permafluor (Beckman-Coulter, Marseille, France) and viewed by fluorescence microscopy.

2.6. Western blotting

Cells were treated with 40 μM BSA or AGE-BSA for 24 h and cell lysates prepared in 50 mM HEPES, 0.05 M NaCl, 0.05% Tween 20, 1% Triton X-100, centrifuged at 12,500 rpm for 1 min and supernatants stored at −20 °C in non-reducing sample buffer until use. Proteins (100 μg/sample) were separated by SDS-10% PAGE, transferred to nitrocellulose and Western blotted with anti-cox 1 (Cell Signalling, Boston, MA), anti-cox 2 (BD Transduction Laboratories, Franklin Lakes, NJ) or anti-β actin (1/1000) in 1% BSA/TBST (50 mM Tris base, 0.15 M NaCl, 0.1% Tween 20, pH 7.4) and proteins detected using enhanced chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL) and exposure to X-ray film for 5 min.

2.7. Thin layer chromatography

Cells grown in 6-well plates were labelled in RPMI containing antibiotics, glutamine, 0.5 g/l BSA and 0.75 μCi/ml [^{3}H]-myo-inositol for 48 h. Cells were treated with BSA or AGE-BSA (40 μM) for 15 min. Cells were scraped into 0.75 ml stop solution (methanol/HCl, 10:1). Water (0.75 ml) and chloroform (1.5 ml) were added before vortexing for 30 s and the top phase removed after centrifugation (1800×*g*, 5 min). Backwash (1 ml of methanol/1 M HCl, 1:1) was added and

samples vortexed for 20 s. The chloroform phase was evaporated to dryness and spotted onto oxalate-coated thin layer chromatography plates (silica gel 60 Merck, Darmstadt, Germany) in 50 µl spotting buffer (chloroform/methanol/10 mM HCl, 20:10:1). Plates were run in 141 ml chloroform/110 ml methanol/23 ml water/10 ml NH_4OH. Phospholipid markers were stained in iodine vapour. Plates were exposed to X-ray film at −80 °C.

2.8. ROS assay

Intracellular reactive oxygen species (ROS) were detected by the oxidation of 2′, 7′-dichlorofluoroscin (DCFH) to the fluorescent product 2′, 7′-dichlorofluoroscein (DCF). Cells were cultured subconfluently on coverslips in SFM for 24 h and then treated with 40 µM BSA or AGE-BSA ± the following inhibitors of arachidonic acid metabolism: 10 µM proadifen hydrochloride, 8.9 nM HET0016, 2.8 µM NDGA or 1 µM indomethacin. After 24 h, 1.5 µl/well of DCFH (3 mg/ml) was added and cells incubated for 5 min at 37 °C. Cells were washed twice with PBS and 3 fluoresecent microphotographic images per treatment were analysed using IMAGE J (http://rsb.info.nih.gov/ij/index.html). Microscope and camera settings were identical within experiments.

2.9. ^{3}H-arachidonic acid release

Cells in GM were cultured subconfluently in 24-well plates. The medium was changed to SFM with 0.5 g/l fatty acid free BSA and cells loaded with 2.5 µCi/well of ^{3}H-arachidonic acid (PerkinElmer, MA) for 24 h. After washing twice in Hank's buffered saline solution (HBSS), cells were incubated in fresh SFM and pretreated with the following inhibitors for 30 min: 10 µM quinacrine dihydrochloride, 2.5 µM HELSS, 1 µM manoalide or 3.5 mM neomycin. Cells were then treated with 40 µM BSA or AGE-BSA for 1 h after which conditioned media were collected, centrifuged at 10,000×*g* for 2 min and mixed with 2.5 ml scintillant (Insta-gel plus, PerkinElmer, Boston, MA) prior to counting in a beta counter.

2.10. 4,5-dimethylthiazol-2-yl-) 2,5-diphenyltetrazolium bromide (MTT) assay

Viable cell number was determined with a colorimetric assay which measures the reduction of MTT by living cells to a blue, water insoluble formazan product. Briefly, LLC-PK1 cells were plated in 96-well culture dishes in 100 µl of GM per well. After 24 h, confluent cells were treated with 40 µM BSA or AGE-BSA in SFM. After 1 or 24 h, MTT (10 µl/well of 5 mg/ml) was added to each well for 4 h at 37 °C. The medium was removed and precipitated formazan was solubilised in 40 mM HCl/isopropanol (100 µl/well) on a plate shaker at room temperature for 30 min. Absorbance at 540 nm was measured in a spectrophotometer. Assays were performed in triplicate within each experiment.

2.11. NKA activity assay

Subconfluent cells were incubated in 12- or 24-well plates in SFM for 24 h and pretreated with 60 nM $cPLA_2\alpha$ inhibitor or 3.5 mM neomycin for 15 min before treatment with 20 µM BSA or AGE-BSA for 30 min. To measure ouabain-sensitive [$^{86}Rb^+$] transport, replicate wells were also treated with 1 mM ouabain hydrate for 30 min at 37 °C. After washing in HBSS, cells were incubated in HBSS with 10 mM HEPES and 1 µCi/ml [$^{86}Rb^+$] (PerkinElmer, Boston, MA) for 10 min at 37 °C. Cells were then washed three times with ice-cold HBSS and lysed in 0.2 ml/well of 3% SDS. Cell lysates were mixed with 2.5 ml scintillant, counted in a beta counter and counts normalised to the protein content of replicate samples. NKA-mediated transport activity was calculated by subtracting the uptake observed in cells preincubated with ouabain from the total uptake. [$^{86}Rb^+$] uptake by LLC-PK1 and HK2 cells was linear for up to 1 h.

2.12. Biotinylation of cell surface proteins

Cells were grown overnight in SFM in Petri dishes. After pretreatment with 60 nM $cPLA_2\alpha$ inhibitor or 3.5 mM neomycin for 15 min, cells were treated with 20 µM BSA or AGE-BSA, trypsinised and washed three times in PBS, pH 8.0. Cells were incubated with 1 mg/ml sulfo-NHS-biotin (Thermoscientific, Rockford, IL) for 30 min at RT, washed three times in 100 mM glycine/PBS and lysed in RIPA buffer (50 mM Tris–HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, pH 7.4) containing a protease inhibitor cocktail (Roche, IN). Lysates were centrifuged at 10,000×*g* for 30 s. Protein (10 mg) was mixed with 60 µl 50% (w/v) streptavidin agarose beads in 0.5 ml RIPA buffer and incubated overnight at 4 °C with mixing. After washing three times in IP buffer (20 mM Tris base, 50 mM NaCl, 0.1% Tween 20 pH 7.4) containing protease inhibitor cocktail, beads were resuspended in non-reducing sample buffer and proteins separated by SDS-8%-PAGE. After transfer, nitrocellulose membranes were probed with an anti-NKA α subunit antiserum (1/5000, Santa Cruz) in 5% skim milk/PBS.

2.13. Co-immunoprecipitation

Cells were grown overnight in Petri dishes in SFM and then treated with 60 nM $cPLA_2\alpha$ inhibitor or 3.5 mM neomycin for 15 min prior to addition of 20 µM BSA or AGE-BSA for 30 min. Cells were scraped into RIPA buffer and 1 mg protein per sample was immunoprecipitated with 1 µl anti-α-adaptin (clone 100/2) or 2.5 µl anti-β2 adaptin (Santa Cruz) antisera overnight at 4 °C in 40 µl of a 50 (v/v)% slurry of protein A sepharose beads (Amersham Biosciences) at 4 °C for 2 h. After washing three times in IP buffer, non-reducing sample buffer was added and proteins separated by SDS-8%-PAGE. After transfer, membranes were probed with anti-NKA α subunit antiserum (1/5000) in 5% skim milk/PBS or anti-α-adaptin (1/5000 anti-β2 adaptin (1/1000) antisera in 1% BSA/TBST.

2.14. PIP5Kγ siRNA

The PIP5Kγ siRNA (Dharmacon, Thermo Scientific, CO) sequence was designed from conserved human/mouse sequences [31] but did not knock down PIP5Kγ in porcine LLC-PK1 cells (results not shown). We therefore used human HK2 cells for siRNA experiments. Control non-targeting pool or PIP5Kγ siRNA (200 nM) was pre-incubated with Dharmafectene 1 (8 µl/ml, Dharmacon) in 0.2 ml SFM for 30 min before addition to subconfluent HK2 cells in 0.6 ml GM in one well of a 6-well plate. After 48 h, cells were seeded subconfluently into 24-well plates. After 24 h, the medium was changed to SFM for 24 h before NKA activity assays were performed as described above.

2.15. Statistics

Data were analysed by *t*-test or ANOVA. Post-hoc analyses were performed using Fisher's protected least significant difference test. Results are expressed as the mean ± SEM. Experiments were repeated between 3 to 12 times as indicated except for Fig. 1B ($n=2$).

3. Results

3.1. Cellular PIP2 is increased by AGE

Immunofluorescence was used to examine the localisation of PIP2 in AGE-BSA-stimulated LCC-PK1 cells. PIP2 levels were increased ~35% in LLC-PK1 cells treated with AGE-BSA, with PIP2 being found in a punctate pattern in AGE-treated cells (Fig. 1A). Thin layer chromatography (TLC) of [^{3}H]-myo-inositol-labelled phospholipids in AGE-BSA-treated LLC-PK1 cells confirmed a ~50% increase in cellular PIP2 levels (Fig. 1B).

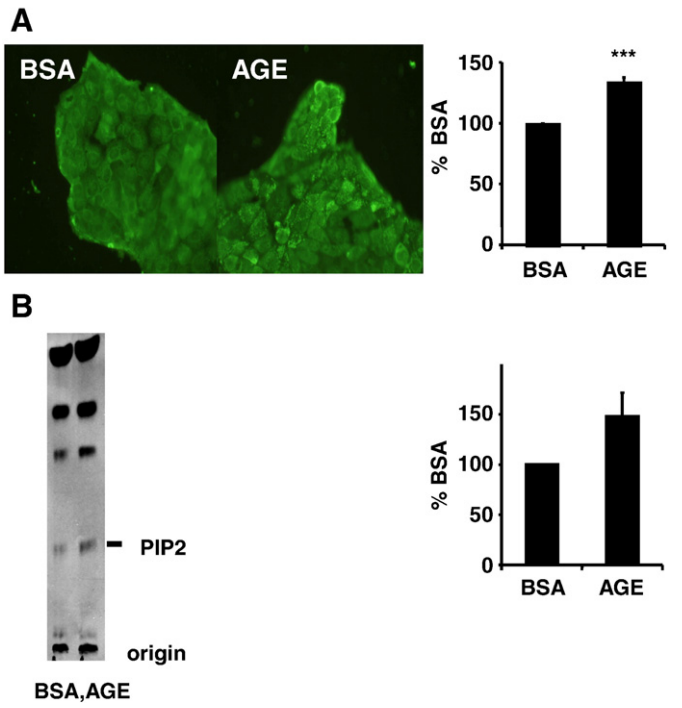

Fig. 1. AGEs increase PIP2 levels. A LLC-PK1 cells were treated with 40 μM BSA or AGE-BSA for 48 h. PIP2 was visualised by immunofluorescence microscopy with anti-PIP2 antiserum. Experiments were performed 3 times with 3 random fields of view/treatment. 400×. ***$p<0.001$ vs BSA B [^{3}H]-myo-inositol-labelled phospholipids from LLC-PK1 cells treated with 40 μM BSA or AGE-BSA for 15 min, were resolved by silica gel thin layer chromatography and autoradiography. $n=2$.

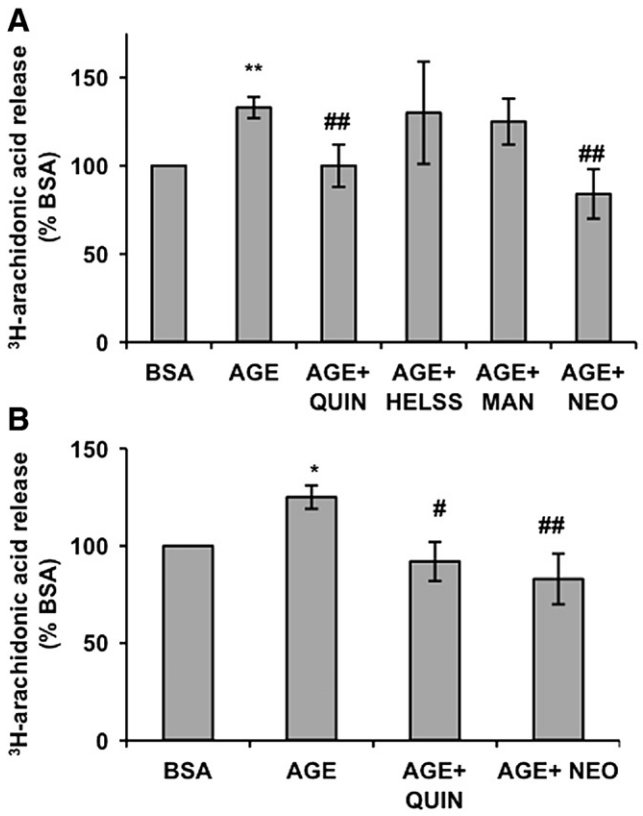

Fig. 2. AGEs induce ^{3}H-arachidonic acid release in proximal tubule cells. ^{3}H-arachidonic acid-loaded A LLC-PK1 or B HK2 cells were pretreated with 10 μM quinacrine dihydrochloride (QUIN), 2.5 μM haloenol lactone (HELSS), 1 μM manoalide (MAN) or 3.5 mM neomycin (NEO) for 30 min before treatment with 40 μM BSA (BSA) or AGE-BSA (AGE) for 1 h. ^{3}H-arachidonic acid released into conditioned media was assayed. Experiments were performed 3–9 times in triplicate and results expressed as a percentage of BSA. *$p<0.05$ and **$p<0.01$ vs BSA, #$p<0.05$ and ##$p<0.01$ vs AGE-BSA.

3.2. AGEs induce ^{3}H-arachidonic acid release in proximal tubule cells

AGEs induce PLA_2 activity and arachidonic acid production in neutrophils [10]. We therefore studied their effect on arachidonic acid metabolism in proximal tubule cells.

In both LLC-PK1 and HK2 cells, AGE-BSA significantly induced the release of ^{3}H-arachidonic acid compared to BSA (Fig. 2A; 133 ± 6%, $p<0.01$, $n=9$ and Fig. 2B; 125 ± 6%, $p<0.05$, $n=5$, respectively). Quinacrine, a $cPLA_2$ inhibitor, significantly inhibited AGE-BSA-induced ^{3}H-arachidonic acid release in LLC-PK1 and HK2 cells (Fig. 2A; 100 ± 12%, $p<0.01$, $n=8$ and Fig. 2B; 92 ± 13%, $p<0.05$, $n=5$, respectively). However, HELSS, a Ca^{2+}-independent PLA_2 inhibitor, and manoalide, a secretory PLA_2 inhibitor, had no effect on AGE-BSA-induced release of ^{3}H-arachidonic acid in LLC-PK1 cells (Fig. 2A; $n=4$). These results suggest that AGEs stimulate PLA_2 activity in both LLC-PK1 and HK2 proximal tubule cell lines. Furthermore, of the various PLA_2 enzymes, $cPLA_2$ appeared to be activated by AGEs.

It was recently shown that $cPLA_2$ is activated by PIP2 [32–34]. We therefore examined the effect of neomycin, a high affinity PIP2-binding molecule [35], on AGE-induced PLA_2 activity. Neomycin significantly inhibited the release of ^{3}H-arachidonic acid induced by AGE-BSA in both LLC-PK1 and HK2 cells (Fig. 2A; 84 ± 14%, $n=4$ and Fig. 2B; 83 ± 10%, $n=5$, respectively, $p<0.01$ for both). These results suggest that PIP2 is involved in AGE-BSA-induced ^{3}H-arachidonic acid release in both proximal tubule cell lines.

3.3. AGEs stimulate arachidonic acid metabolism through cyclooxygenase

Activation of PLA_2 leads to arachidonic acid release and its subsequent oxidation by various downstream enzymes, including cyclooxygenases, lipooxygenases and cytochrome P450 enzymes [11]. Metabolism of arachidonic acid by these enzymes induces the production of reactive oxygen species (ROS). We used this characteristic to identify downstream effectors of AGE-induced arachidonic acid metabolism using specific inhibitors.

AGE-BSA significantly induced ROS production compared to BSA in both LLC-PK1 and HK2 cells (Fig. 3A; 222 ± 29%, $p<0.001$, $n=12$ and Fig. 3B; 159 ± 11%, $p<0.01$, $n=8$, respectively). A similar result was also seen in LLC-PK1 cells treated with 40 μM AGE-RNAse (results not shown), confirming that the effect was due to the glycated moiety and not the specific protein.

Indomethacin, a cox 1/2 inhibitor, significantly inhibited AGE-BSA-induced ROS formation in both LLC-PK1 and HK2 cells (Fig. 3A; 86 ± 13%, $n=3$ and Fig. 3B; 87 ± 12%, $n=6$, respectively, $p<0.001$ for both). In contrast, HET0016 and proadifen, specific cytochrome P450 inhibitors, and NDGA, a lipoxygenase inhibitor, had no effect on AGE-BSA-induced ROS formation in LLC-PK1 cells (Fig. 3A). These results suggest that cox activity is downstream of $cPLA_2$ signalling induced by AGE-BSA in proximal tubule cells.

AGE-BSA-induced ROS production was significantly inhibited by neomycin in both LLC-PK1 and HK2 cells (Fig. 3A; 88 ± 18%, $p<0.01$, $n=3$ and Fig. 3B; 72 ± 35%, $p<0.001$, $n=4$, respectively). These results suggest involvement of PIP2 in AGE-BSA ROS signalling in both LLC-PK1 and HK2 proximal tubule cells.

3.4. NKA surface expression and activity is decreased by AGEs in a time- and dose-dependent manner

LLC-PK1 cells were treated with 20 μM BSA or AGE-BSA for up to 1 h. Cells were stained for NKA α subunit protein and examined by fluorescence microscopy (Fig. 4A). In LLC-PK1 cells, NKA staining was localised predominantly at the cell periphery [36]. Compared with

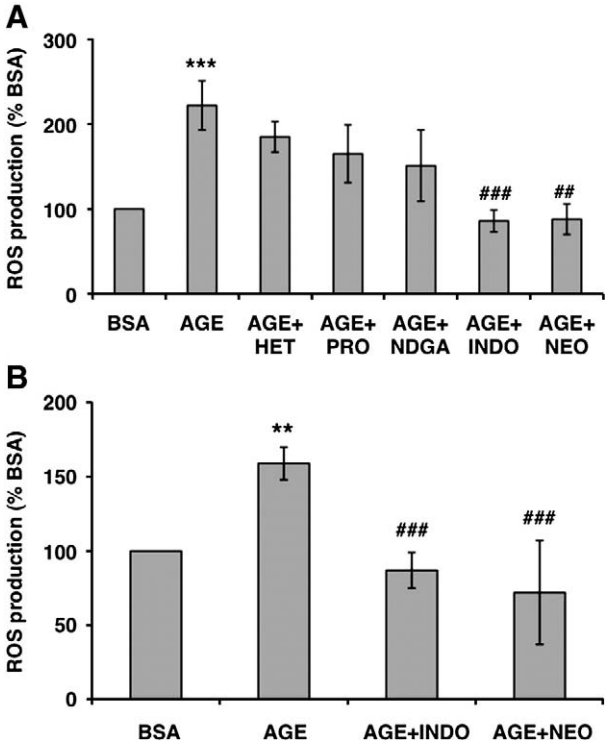

Fig. 3. Cyclooxygenase is a determinant of ROS production and arachidonic acid metabolism by AGEs. A LLC-PK1 or B HK2 cells were treated with 40 μM BSA (BSA) or AGE-BSA (AGE) ± 8.9 nM HET0016 (HET), 10 μM proadifen (PRO), 2.8 μM nordihydroguaiaretic acid (NDGA), 1 μM indomethacin (INDO) or 3.5 mM neomycin (NEO) for 24 h. DCFH was added and ROS production measured by fluorescence microscopy and quantified by Image J. Experiments were performed 3–12 times with 3 random fields of view/treatment and results expressed as a percentage of BSA. $^{**}p<0.01$ and $^{***}p<0.001$ vs BSA, $\#\#p<0.01$ and $\#\#\#p<0.001$ vs AGE-BSA.

BSA, AGE-BSA decreased staining for NKA at the cell periphery by 15 min with complete disappearance of NKA from the cell surface by 60 min. This rapid effect suggests that AGEs may stimulate NKA endocytosis. An MTT assay was performed to exclude toxic effects of AGEs on LLC-PK1 cells. This showed that AGE-BSA had no effect on viable cell number compared to vehicle-treated or BSA-treated cells after 1 or 24 h (Fig. 4B).

AGE-BSA reduced surface expression of NKA α subunit (Supplementary Fig. 1) and NKA activity as assessed by [$^{86}Rb^{+}$] uptake (Fig. 5) in a dose-dependent manner.

3.5. NKA surface expression is decreased by different AGE preparations

To confirm that the glycated moiety of AGE-BSA was responsible for NKA inhibition, we tested a number of different AGE preparations. AGE-RNase and MGO-BSA also reduced cell surface expression of NKA (Fig. 6) (at concentrations as low as 5 μM, results not shown). Importantly, endogenously glycated human albumin also reduced cell surface expression of NKA, confirming that this AGE action is physiologically relevant.

3.6. AGEs inhibits NKA surface expression via cPLA$_2$α and cox 2

NKA is downregulated in various cell types in diabetes and diabetic models [18–21,37,38]. Furthermore, arachidonic acid metabolites inhibit NKA activity in proximal tubule cells [39–42]. We therefore examined the effect of AGE-induced cPLA$_2$ activity on NKA activity in proximal tubule cells.

Pre-treatment of LLC-PK1 cells with a specific cPLA$_2$α inhibitor reversed the inhibitory effect of AGE-BSA on NKA surface expression (Fig. 7A). Indomethacin, an inhibitor of cox 1/2, and ns-398, a specific cox 2 inhibitor, but not sc-560, a cox 1 inhibitor, reversed the inhibitory effect of AGE-BSA on NKA surface expression (Fig. 7A). Arachidonic acid and, to a lesser extent, PGE$_2$, products of cPLA$_2$ and cyclooxygenase 2 respectively, also decreased expression of surface NKA in LLC-PK1 cells. These results indicate that AGE-BSA inhibits surface expression of NKA via a pathway involving cPLA$_2$α and cox 2.

Both cox 1 and 2 proteins were detected in HK2 cells by Western blotting (Fig. 7B). However, AGE-BSA increased cox 2 but not cox 1 levels (Fig. 7B). Cox 1 and cox 2 were not detected in LLC-PK1 cells using these antibodies, most likely due to species differences.

3.7. AGEs inhibit the surface expression of biotinylated NKA and this is reversed by cPLA$_2$α inhibitor and neomycin

AGE-BSA did not affect total cell NKA protein levels after 24 h treatment (Fig. 8A (i)). However, AGE-BSA decreased cell surface expression of NKA after 30 min as determined by pulldown of biotinylated surface proteins followed by Western blotting (Fig. 8A (ii)). This effect was reversed by pre-treatment of cells with a cPLA$_2$α inhibitor or neomycin (Fig. 8B). Neomycin also reversed AGE-BSA-inhibition of surface expression of NKA as determined by immunofluorescence (Fig. 8C). These experiments confirm the inhibitory effect of AGE-BSA on cell surface expression of NKA and the involvement of cPLA$_2$α and PIP2.

3.8. Regulation of NKA activity by AGE-induced cPLA$_2$α and PIP2

AGE-BSA significantly inhibited NKA activity compared to BSA control in both LLC-PK1 and HK2 cells (Fig. 9A; 80 ± 3%, $n=12$ and Fig. 9B; 66 ± 6%, $n=7$, respectively, $p<0.01$ for both). The cPLA$_2$α inhibitor reversed the AGE-BSA-induced inhibition of NKA activity in both LLC-PK1 and HK2 cells (Fig. 9A; 116 ± 7%, $n=6$, $p<0.05$ and Fig. 9B; 111 ± 12%, $n=5$, $p<0.01$, respectively). Inhibiting PIP2 with neomycin also significantly reversed the AGE-BSA-induced inhibition of NKA activity in both cell lines (Fig. 9A; 96 ± 11%, $n=9$ and Fig. 9B; 100 ± 16%, $n=4$, respectively, $p<0.05$ for both).

3.9. Src inhibition reverses the AGE-induced decrease in NKA

In LLC-PK1 cells, clathrin- and caveolin-mediated endocytosis of NKA stimulated by ouabain involves Src signalling [43–45]. AGEs activate Src [46] so we examined a possible role for Src in AGE-induced inhibition of NKA. AGE-BSA-induced ROS production was significantly lower in LLC-PK1 cells pretreated with PP2, a Src inhibitor, than in cells treated with PP3, a negative control peptide (Fig. 10A; 84 ± 9%, $p<0.001$ vs. AGE + PP3 176 ± 28%, $n=3$). Similar findings were observed in HK2 cells (not shown). PP2 but not PP3 also reversed the loss of surface expression of NKA by AGE as determined by immunofluorescence (Fig. 10B) and biotinylation (Fig. 10C) studies, respectively. These results suggest that Src is involved in AGE-BSA-induced inhibition of surface expression of NKA.

3.10. AGEs increase binding of PIP5Kγ and NKA to the α-adaptin subunit of the clathrin adaptor protein AP-2

Endocytosis of NKA has been reported to be clathrin-dependent [47]. We therefore examined the binding of NKA to the α-adaptin subunit of the AP-2 clathrin adaptor complex and found that AGE-BSA increased NKA binding after 30 min (Fig. 11A).

AGE-BSA increased cellular PIP2 levels (Fig. 1), suggesting activation of PIP5K. All three isoforms of PIP5K, α, β and γ, bind AP-2 or are involved in clathrin-mediated endocytosis [48–50]. However, AP-2 and clathrin specifically bind and/or activate PIP5Kγ661 to

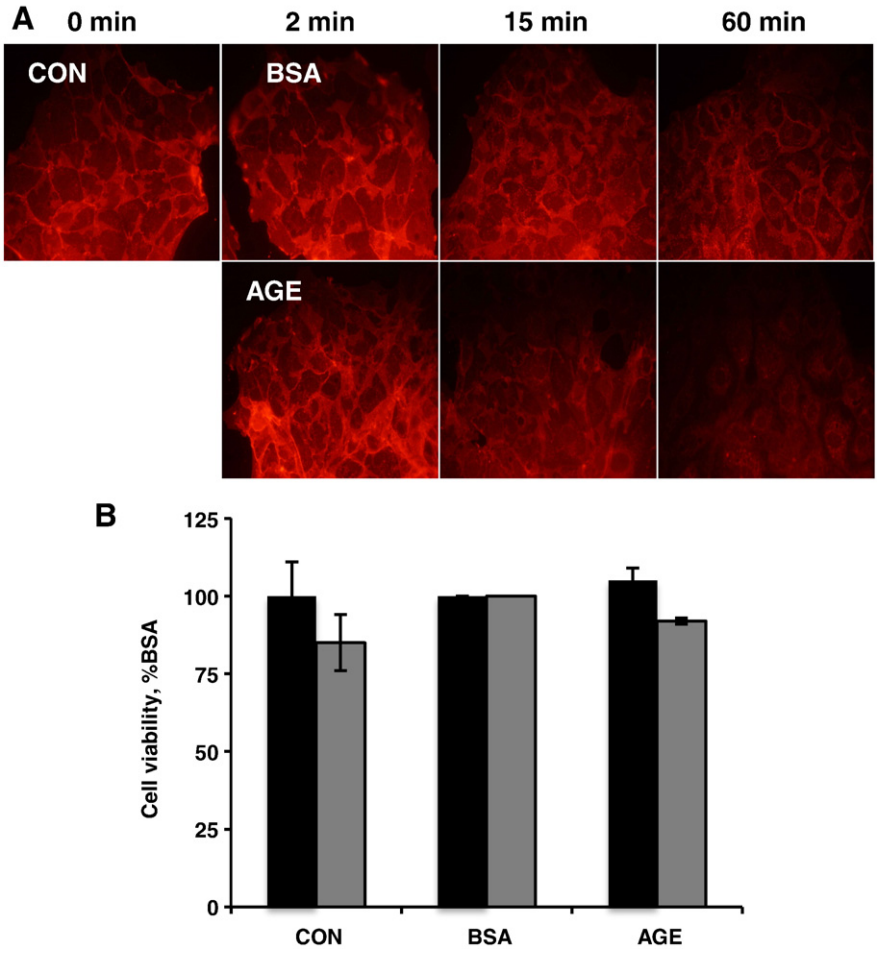

Fig. 4. AGEs inhibit the surface expression of NKA in a time-dependent manner and do not affect cell viability. A LLC-PK1 cells were incubated with PBS vehicle (Con), 20 μM BSA or AGE-BSA for various time points. Cells were fixed and NKA α subunit visualised by immunofluorescence microscopy. 400×. Experiments were performed 3 times with 3 random fields of view/treatment. B LLC-PK1 cells were incubated with PBS vehicle (Con), 40 μM BSA or AGE-BSA for 1 (black) or 24 (grey) hours. Viable cell numbers were determined by MTT assay. Experiments were performed 3 times and expressed as a percentage of BSA control.

regulate endocytosis [51–53]. We therefore studied whether PIP5Kγ was involved in the stimulation of NKA endocytosis by AGE-BSA. AGE-BSA enhanced binding of PIP5Kγ, in addition to NKA, to the AP-2 α-adaptin subunit (Fig. 11A), but AGE-BSA had no effect on binding of PIP5Kγ to the AP-2 β2-adaptin subunit (Fig. 11B).

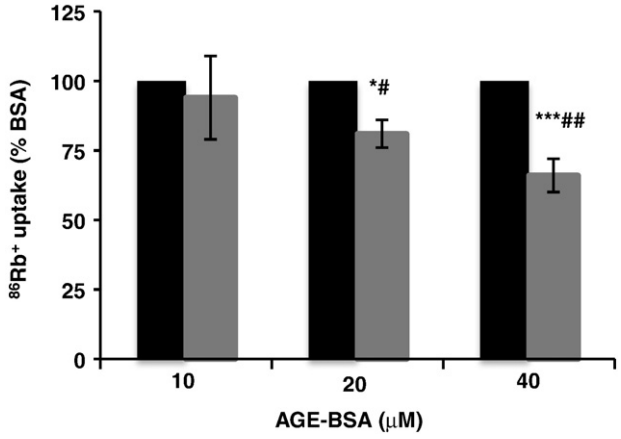

Fig. 5. AGEs inhibit NKA activity in a dose-dependent manner. LLC-PK1 cells were incubated with 10–40 μM BSA (black) or AGE-BSA (grey). After washing, cells were incubated in HBSS with 10 mM HEPES and 1 μCi/ml [$^{86}Rb^+$] for 10 min at 37 °C. Cell lysates were counted in a beta counter and counts normalised for protein content. Experiments were performed 3–4 times with 3 replicates/treatment and results expressed as a percentage of BSA for a given treatment. *$p<0.05$ and ***$p<0.001$ vs. BSA, #$p<0.05$ and ##$p<0.01$ vs. 10 μM AGE-BSA.

3.11. PIP5Kγ is essential for AGE induced inhibition of NKA activity

To determine the role of PIP5Kγ in AGE-induced inhibition of NKA activity, we used siRNA to knock down expression by ~90% (Fig. 12A). AGE-BSA significantly inhibited NKA activity in control siRNA-transfected HK2 cells compared to BSA (Fig. 12B; 59 ± 8%, $n=7$, $p<0.001$). In contrast, AGE-BSA had no significant effect on NKA activity in PIP5Kγ siRNA-transfected cells (Fig. 12B; 84 ± 4%, $n=7$). These results suggest that AGE-BSA-induced inhibition of NKA activity in HK2 cells is dependent on PIP5Kγ.

4. Discussion

AGEs are implicated in the development of diabetic nephropathy. Disruption of NKA activity is a feature of a number of renal pathologies. In particular, NKA activity is decreased in many tissues including kidney, sciatic nerve and red blood cells in diabetic patients and animals. Erythrocyte NKA activity is decreased in patients with types 1 and insulin-requiring type 2 diabetes and may be related to C-peptide levels [17–23]. In patients with uncontrolled type 1 diabetes, this decrease correlates with hyperglycemia and is corrected by its normalisation. In streptozotocin (STZ)-diabetic rats, NKA activity is decreased by ~40% in erythrocytes, sciatic nerve and kidney [37]. NKA activity is also reduced

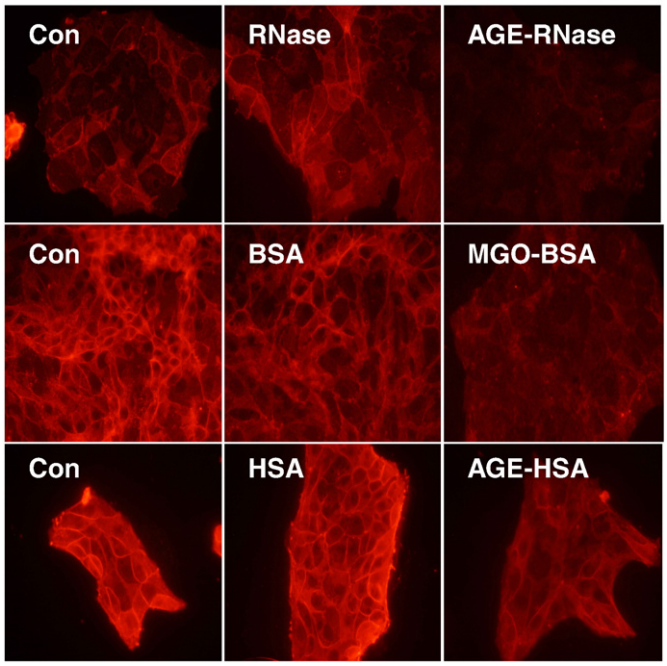

Fig. 6. Various AGEs inhibit the surface expression of NKA. LLC-PK1 cells were incubated with PBS control (Con), 40 μM RNase, AGE-RNase, BSA, MGO-BSA, human normal or glycated albumin for 30 min. Cells were fixed and NKA α subunit visualised by immunofluorescence microscopy. 400×. Experiments were performed 3 times with 3 random fields of view/treatment.

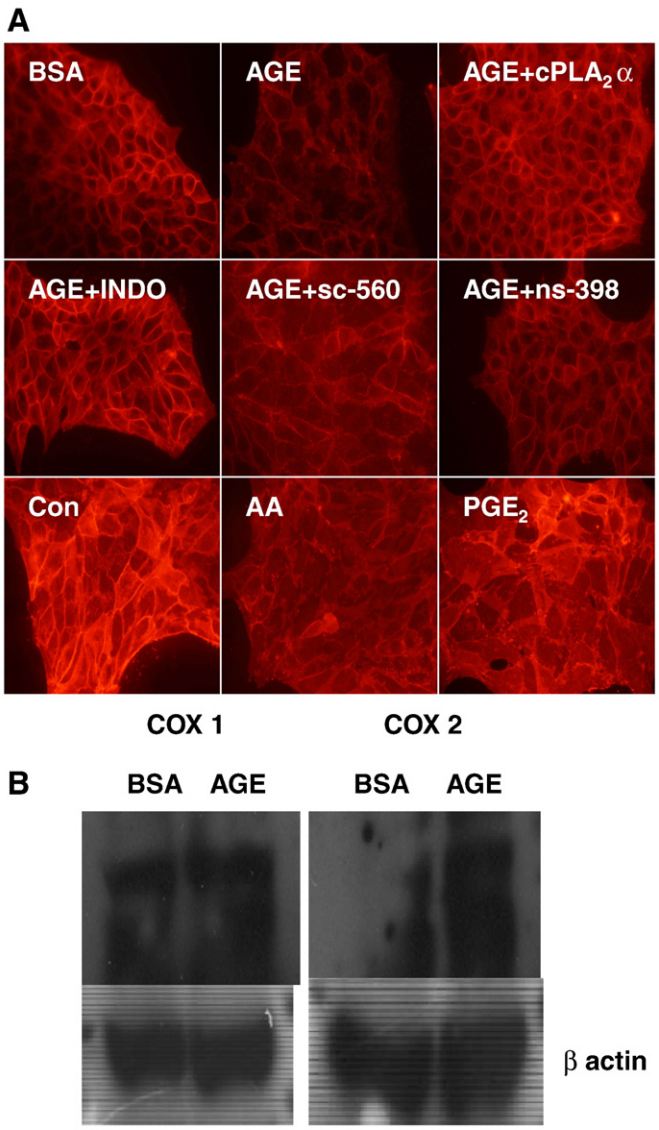

Fig. 7. Role of $cPLA_2\alpha$ and cox 2 in AGE-induced inhibition of surface NKA. A LLC-PK1 cells were pre-incubated with 60 nM $cPLA_2\alpha$ inhibitor, 1 μM indomethacin, 10 nM sc-560, or 32 μM ns-398 for 15 min. and then 20 μM BSA or AGE-BSA for 30 min. LLC-PK1 cells were also treated with 0.2% ethanol vehicle (Con), 30 nM arachidonic acid or 20 μg/ml PGE_2. Cells were fixed and NKA α subunit visualised by immunofluorescence microscopy 400×. Experiments were performed 3 times with 3 random fields of view/treatment. B HK2 cells were treated with 40 μM BSA or AGE-BSA for 24 h. Lysates were collected and proteins separated by SDS-10%-PAGE and Western blotting performed with cox 1 or cox 2 and β actin antisera. Experiments were performed 3 times.

in the medullary thick ascending limb in STZ-diabetic rats after 12 weeks [38]. NKA itself is glycated in kidney and erythrocytes and is thought to contribute to reduced lens ion transport that occurs during the development of diabetic cataracts [54,55]. Our studies suggest an additional mechanism for decreased NKA activity in proximal tubule cells during diabetes, namely that AGEs inhibit NKA activity by increasing the activities of $cPLA_2\alpha$ and PIP5Kγ to reduce cell membrane expression of NKA via endocytosis. Importantly, endogenously glycated human albumin also decreased cell surface NKA expression, illustrating the physiological relevance of our findings.

The physiological activation of PLA_2 and cyclooxygenase pathways is important for normal renal function as these modulate salt and water reabsorption [56]. Renal eicosanoid levels are altered in diseases such as diabetes and may contribute to nephropathy. AGEs stimulate $cPLA_2$ activity in neutrophils [10]. We found that AGEs induced $cPLA_2\alpha$ activity in proximal tubule cells as indicated by (i) stimulation of ^{3}H-arachidonic acid release and its attenuation by quinacrine, (ii) prevention of AGE-BSA inhibition of cell membrane NKA by a $cPLA_2\alpha$ inhibitor, (iii) inhibition of cell membrane NKA by arachidonic acid and (iv) attenuation of AGE-BSA-induced inhibition of NKA activity by a $cPLA_2\alpha$ inhibitor. Our findings are consistent with the modulation of NKA by arachidonic acid and its metabolites that has been shown previously in various nephron segments [39–42].

High glucose levels increase PLA_2 activity and PGE_2 generation in mesangial cells and increase PGE_2 and decrease NKA activity in cerebral endothelial cells [57,58]. In contrast, other studies have shown that AGE-BSA inhibits prostacyclin production in human endothelial cells and PGE_2 secretion by human proximal tubular cells [59,60]. These contrasting findings suggest that AGEs and glucose may have different effects or that effects are cell-type specific.

We found AGEs induced cyclooxygenase activity as indicated by the sensitivity of AGE-induced ROS formation to indomethacin. Involvement of cox 2 was subsequently identified by the (i) attenuation of AGE-BSA inhibition of cell membrane expression of NKA by ns-398, (ii) inhibition of cell membrane expression of NKA by PGE_2 and (iii) upregulation of cox 2 but not cox 1 protein by AGE-BSA. In monocytes, cox 2 mRNA is increased by AGEs, high glucose or diabetes, while overexpression of RAGE, the receptor for AGEs, is associated with enhanced cox 2 protein expression in diabetic plaque macrophages [61–63]. However, high glucose inhibits NKA activity in pancreatic β-cells via the lipoxygenase pathway [64] which shows that different pathways could play a role in the regulation of NKA activity in different cells. Other stimuli, including endothelin, also inhibit NKA activity in proximal tubules via cox [41,65–67] whereas parathyroid hormone and angiotensin II both inhibit NKA activity in the proximal tubule via the cytochrome P450 pathway [39,40]. Additionally, PGE_2 inhibition of NKA surface expression appeared less complete than that by arachidonic acid, suggesting the possible involvement of other cox metabolites such as TXA_2, PGI_2, PGF_2 or PGD_2. This possibility requires further investigation.

AGEs increased cellular PIP2 levels in the current study. Neomycin, which sequesters and inhibits PIP2 [35], inhibited both AGE-induced

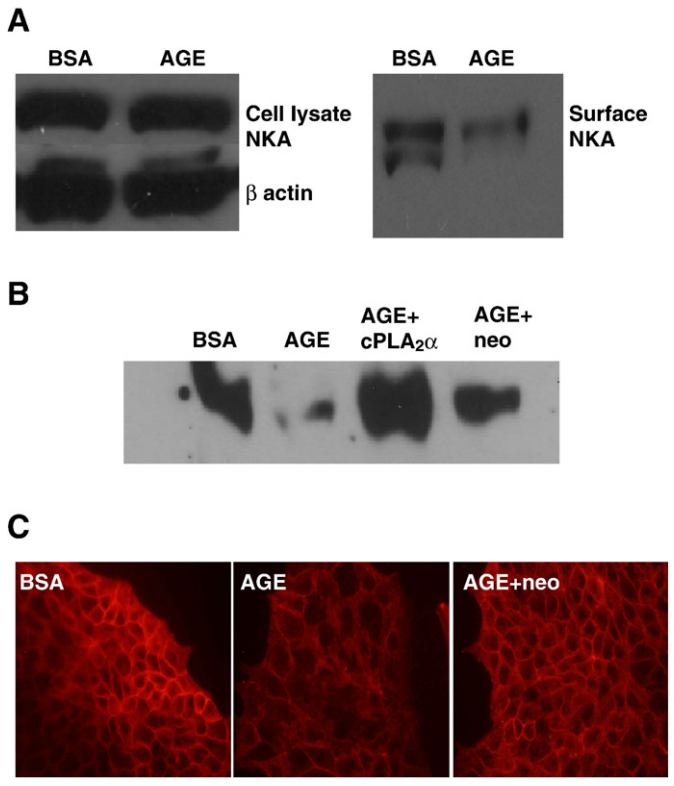

Fig. 8. AGEs inhibit surface expression of NKA: role of $cPLA_2\alpha$ and PIP2. A LLC-PK1 cells were incubated with 20 μM BSA or AGE-BSA (i) for 24 h and whole cell lysates collected or (ii) for 30 min and cell surface proteins biotinylated, isolated with streptavidin sepharose beads, separated by SDS-10%-PAGE, blotted and probed with anti-NKA α subunit antiserum. Experiments were performed 3 times. B LLC-PK1 cells were pre-incubated with 60 nM $cPLA_2\alpha$ inhibitor or 3.5 mM neomycin for 15 min and then 20 μM BSA or AGE-BSA for 30 min. Cell surface proteins were biotinylated and analysed as in **A** (ii). Experiments were performed 3 times. C LLC-PK1 cells were incubated with 40 μM BSA or AGE-BSA ± 3.5 mM neomycin (NEO) for 30 min. Cells were fixed and NKA α subunit visualised by immunofluorescence microscopy 400×. Experiments were performed 3 times with 3 random fields of view/treatment.

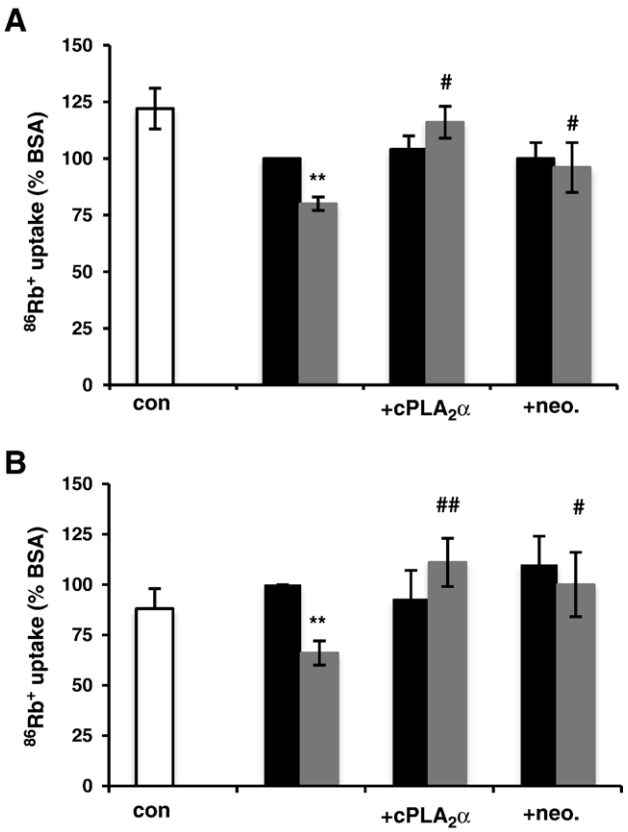

Fig. 9. AGEs inhibit NKA activity: role of $cPLA_2\alpha$ and PIP2. A LLC-PK1 or B HK2 cells were pre-incubated with 0.01% ethanol vehicle, 60 nM $cPLA_2\alpha$ inhibitor ($cPLA_2\alpha$) or 3.5 mM neomycin (neo) for 15 min and then 20 μM BSA (black), AGE-BSA (grey) or an equal volume of PBS (white) for 30 min. After washing, cells were incubated in HBSS with 10 mM HEPES and 1 μCi/ml [$^{86}Rb^+$] for 10 min at 37 °C. Radioactivity was counted in a beta counter and normalised for protein content. Experiments were performed 4–12 times with 3 replicates/treatment and results expressed as a percentage of BSA control. $^{**}p<0.01$ vs. BSA, $\#p<0.05$ and $\#\#p<0.01$ vs AGE-BSA.

arachidonic acid release and ROS production. Since PIP2 binding to $cPLA_2$ dramatically increases its activity and membrane association [32–34], it is possible that AGEs also stimulate $cPLA_2$ activity by activating PIP5Kγ and upregulating PIP2 levels.

Liu et al. found that Src activity is essential for the inhibition of surface expression of NKA in LLC-PK1 cells by ouabain via clathrin- and caveolin-mediated endocytosis [43–45]. We found that inhibiting Src reversed the AGE-induced decrease of surface expression of NKA in LLC-PK1 cells as determined by immunofluorescence and biotinylation. Pertinently, AGEs activate Src in other cell types [46]. Liu et al. found Src was recruited to caveolin, clathrin and endosomes upon ouabain stimulation [44,45]. We showed that AGEs increase binding of NKA to the α-adaptin subunit of the clathrin endocytic adaptor complex, AP-2, implicating the involvement of clathrin-mediated endocytosis. Future studies aimed at confirming our hypothesis that AGEs inhibit NKA by clathrin-mediated endocytosis require showing that subcellular localisation of NKA to early and late endosomes and clathrin vesicles. Possible Src recruitment to clathrin vesicles also warrants further study.

Clathrin-mediated endocytosis of some cargoes requires PIP2-dependent membrane recruitment of AP-2 adaptor complexes [68]. We found increased binding of PIP5Kγ to the AP-2 α adaptin subunit and also found that neomycin prevented AGE-induced inhibition of NKA surface expression and activity. Neomycin inhibits clathrin/AP-2 recruitment to the cell membrane [69–71], suggesting it may have interfered with AGE-induced NKA endocytosis in this manner.

The α- and μ2-adaptin subunits of the α,β2,μ2,σ2 tetrameric AP-2 complex contain PIP2 binding sites which allows them to interact with the plasma membrane and regulate clathrin-mediated endocytosis [68,69,72]. PIP2 elevated by PIP5Kβ and γ isoforms is implicated in AP-2 recruitment to the cell membrane [49,50]. In contrast, PIP5Kγ661 enhances AP-2-regulated endocytosis through its μ2-subunit without affecting cellular PIP2 levels [53]. We found AGEs increased overall cellular levels of PIP2 and increased binding of PIP5Kγ to the AP-2 α-adaptin subunit. PIP2 may be involved in corecognition of protein cargo by AP-2, AP-2 relocalisation to the cell membrane [49,50,72] or exert direct effects on increasing clathrin pit formation [73].

Several groups have shown that AP-2 itself activates PIP5Kγ [50,53,74]. We observed increased binding of PIP5Kγ to the AP-2 α-adaptin subunit, which is consistent with previous studies showing co-immunoprecipitation of endogenous PIP5K with α- and β-adaptins, although this interaction was thought to be indirect [74,75]. Others have shown that PIP5K is activated by AP-2 after its association with cargo proteins via the μ2-adaptin subunit [75], whereas PIP5Kγ661 is activated by AP-2 through β2- or μ2-adaptins [53,74].

Isoform specific functions of PIP5K contribute to specific cell functions at the plasma membrane such as the formation of focal adhesions and clathrin adaptor recruitment and receptor internalisation [49,76]. PIP5Kβ regulates constitutive endocytosis while α and γ isoforms regulate stimulated endocytosis in nonneuronal and

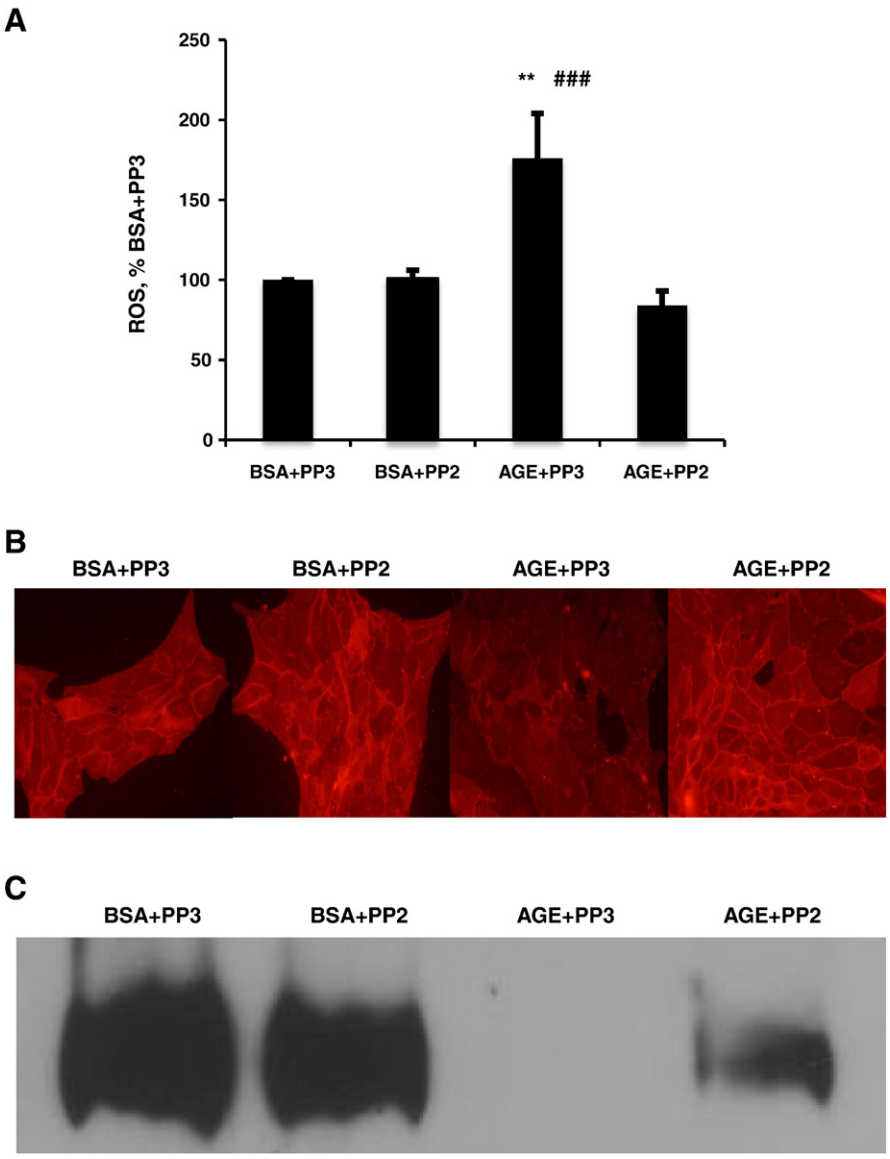

Fig. 10. Src inhibition reverses the inhibitory effect of AGE on NKA. LLC-PK1 cells were pretreated with 100 nM PP3 or PP2 for 15 min, then treated with 40 μM BSA (BSA) or AGE-BSA (AGE) for 30 min. A DCFH was added and ROS production measured by fluorescence microscopy and quantified by Image J. Experiments were performed 3 times with 3 random fields of view/treatment and results expressed as a percentage of BSA + PP3. $^{**}p<0.01$ vs. BSA + PP3, $^{###}p<0.001$ vs. AGE-BSA + PP2. B Cells were fixed and NKA α subunit visualised by immunofluorescence microscopy. 400×. Experiments were performed 3 times with 3 random fields of view/treatment. C Cell surface proteins were biotinylated, isolated with streptavidin sepharose beads, separated by SDS-8%-PAGE, blotted and probed with anti-NKA α subunit antiserum. Experiments were performed 3 times.

neuronal cells, respectively [31,48–50,77,78]. Few, if any, studies of PIP5K and endocytosis have been reported in kidney cells or in relation to a specific pathological process such as diabetes. However, PIP5Kα enhanced endocytosis of the epithelial sodium channel in a PIP2-dependent manner in cultured kidney collecting duct cells [48]. To our knowledge, our studies are the first to show PIP5Kγ function during endocytosis in a non-neuronal cell line.

Is there evidence that elevated cellular PIP2 may be pathological in diabetes? There are a number of diseases in which PIP2 levels are elevated [79]. One of these is Lowe syndrome, which is characterised by proximal tubule reabsorption defects and dysregulated clathrin-mediated membrane trafficking as a result of dysfunctional phosphoinositide metabolism [79]. PIP2 levels are elevated in proximal tubule cells from patients with Lowe syndrome due to OCRL, a mutated inositol 5-phosphatase. Circulating levels of lysosomal enzymes are also increased, suggesting dysregulated lysosomal vesicular trafficking [79,80]. In patients with diabetes, levels of the urinary lysosomal enzyme, N-acetyl B glucosaminidase (NAG), a marker of proximal tubular injury, correlates with glycated haemoglobin levels [81]. We observed that AGE-BSA increased NAG release by ~40% in LLC-PK1 cells (results not shown), and, together with the above observations in Lowe syndrome, suggests that this may be related to elevated cellular PIP2 levels. Whether diabetic nephropathy is a PIP2-mediated disease warrants further study.

4.1. Conclusions

In summary, our results demonstrate that AGEs stimulate $cPLA_2$, cyclooxygenase and PIP5Kγ/PIP2 activities to inhibit NKA (Fig. 13). AGEs are shown to inhibit NKA surface expression and activity in a time- and dose-dependent manner and this is reliant on $cPLA_2\alpha$, cyclooxygenase 2, Src and PIP2. Inhibition of NKA activity by AGEs is due to increased binding of NKA to the α-adaptin subunit of the clathrin endocytic adaptor complex, AP-2, which would enhance NKA endocytosis from the cell surface. AGEs also increase binding of PIP5Kγ to α-adaptin. Enhanced PIP2 from PIP5Kγ activity may

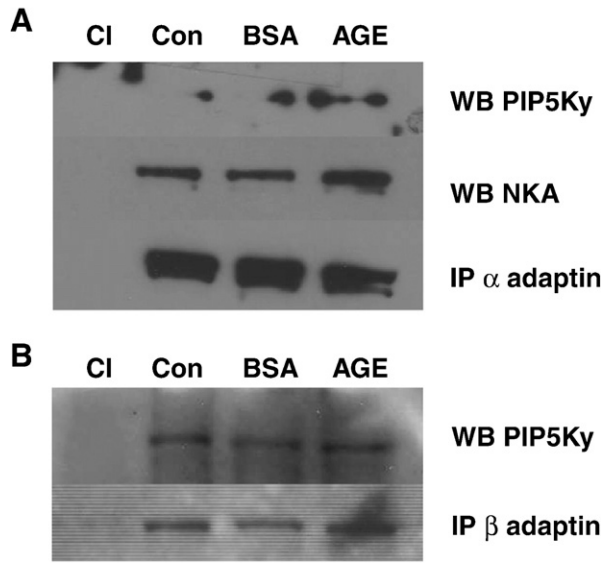

Fig. 11. AGEs increase binding of NKA α subunit and PIP5Kγ to the α-adaptin subunit of the AP-2 adaptor complex. LLC-PK1 cells were incubated with PBS vehicle (Con), 40 μM BSA or AGE-BSA for 30 min. Adaptin proteins in cell lysates were immunoprecipitated with α-, or β2-adaptin antisera and protein A sepharose beads. Normal mouse IgG was used as control (Cl). Proteins were separated by SDS-8%-PAGE, blotted and probed with A anti-α-adaptin, -PIP5Kγ and -NKA α antisera or B anti-β2-adaptin and -PIP5Kγ antisera. Experiments were performed 3–4 times.

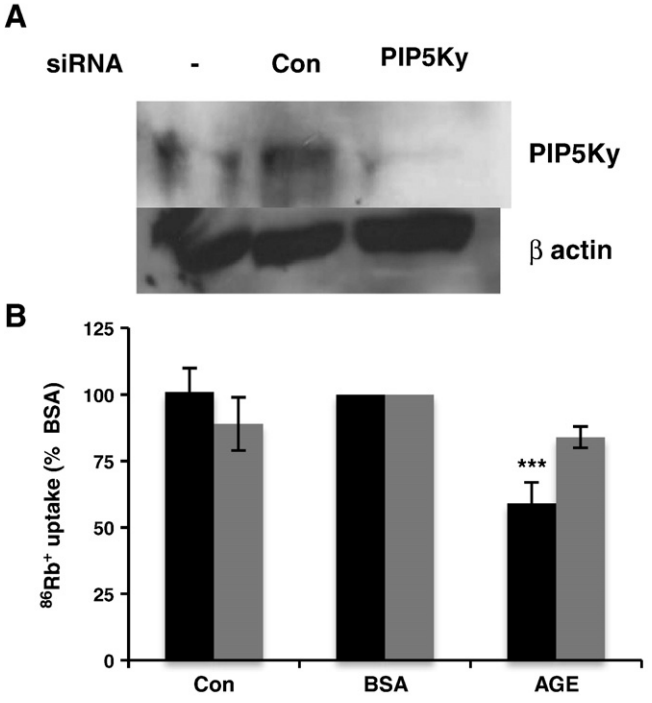

Fig. 12. PIP5Kγ siRNA reverses AGE inhibition of NKA activity. A HK2 cells were transfected with control or PIP5Kγ siRNA for 48 h. Proteins in cell lysates were separated by SDS-8%-PAGE and Western blotting performed with anti-PIP5Kγ and β-actin antisera. B HK2 cells transfected with vector control (black) or PIP5Kγ (grey) siRNA were incubated with PBS vehicle (con), 40 μM BSA or AGE-BSA for 30 min. After washing, cells were incubated in HBSS with 10 mM HEPES and 1 μCi/ml [$^{86}Rb^{+}$] for 10 min at 37 °C. Radioactivity was counted in a beta counter and normalised for protein content. Experiments were performed 7 times with 3 replicates/treatment and results expressed as a percentage of BSA control. ***$p<0.001$ vs. BSA.

facilitate AP-2 localisation to clathrin pits, increase clathrin pit formation, enhance cargo recognition by AP-2 or stimulate $cPLA_2\alpha$ activity. These studies are the first to link AGEs to dysregulation of phosphoinositide metabolism and the first to link a specific pathological process such as diabetes to increased PIP5K activity and enhanced endocytosis of NKA cargo.

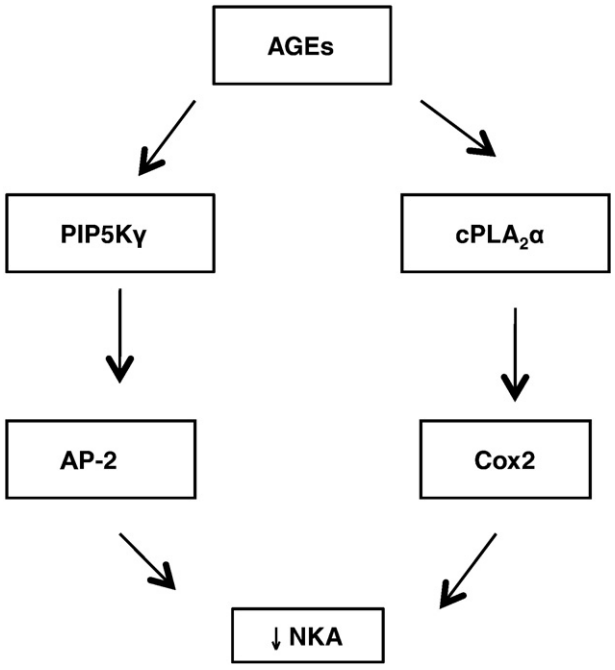

Fig. 13. Postulated pathways by which AGEs inhibit NKA activity in proximal tubule cells.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2010.04.009.

References

[1] H. Vlassara, R. Bucala, S. L, Pathogenic effects of advanced glycosylation: biochemical, biologic and clinical implications for diabetes and aging, Lab. Invest. 70 (1994) 138–151.
[2] P. Beisswenger, Z. Makita, T. Curphey, L. Moore, S. Jean, T. Brinck-Johnsen, R. Bucala, H. Vlassara, Formation of immunochemical advanced glycosylation end products precedes and correlates with early manifestations of renal and retinal disease, Diabetes 44 (1995) 824–829.
[3] A. Bierhaus, M. Hofmann, R. Ziegler, P. Nawroth, AGEs and their interaction with AGE-receptors in vascular disease and diabetes mellitus, Cardiovasc. Res. 37 (1998) 586–600.
[4] H. Vlassara, L. Striker, S. Teichberg, H. Fuh, Y. Li, M. Steffes, Advanced glycation end products induce glomerular sclerosis and albuminuria in normal rats, Proc. Natl Acad. Sci. USA 91 (1994) 11704–11708.
[5] M. Brownlee, H. Vlassara, A. Kooney, P. Ulrich, A. Cerami, Aminoguanidine prevents diabetes-induced arterial wall cross-linking, Science (1986) 1629–1632.
[6] M. Kihara, J. Schmelzer, J. Poduslo, G. Curran, K. Nickander, P. Low, Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology and oxygen free radicals, Proc. Natl Acad. Sci. USA 88 (1991) 6107–6111.

[7] Z. Makita, S. Radoff, E. Rayfield, Z. Yang, E. Skolnik, V. Delaney, E. Friedman, A. Cerami, H. Vlassara, Advanced glycation end products in patients with diabetic nephropathy, N Engl J. Med. 325 (1991) 836–842.

[8] A.F.M.L. Lieuw, V.W. van Hinsbergh, T. Teerlink, R. Barto, J. Twisk, C.D. Stehouwer, C.G. Schalkwijk, Increased levels of N(epsilon)-(carboxymethyl)lysine and N (epsilon)-(carboxyethyl)lysine in type 1 diabetic patients with impaired renal function: correlation with markers of endothelial dysfunction, Nephrol. Dial. Transplant. 19 (2004) 631–636.

[9] M.A. Gallicchio, E.A. McRobert, A. Tikoo, M.E. Cooper, L.A. Bach, Advanced glycation end products inhibit tubulogenesis and migration of kidney epithelial cells in an ezrin-dependent manner, J. Am. Soc. Nephrol. 17 (2006) 414–421.

[10] R. Wong, A. Pettit, P. Quinn, S. Jennings, J. Davies, L. Ng, Advanced glycation end products stimulate an enhanced neutrophil respiratory burst mediated through the activation of cytosolic phospholipase A_2 and generation of arachidonic acid, Circulation 108 (2003) 1858–1864.

[11] R. Adibhatla, J. Hatcher, Phospholipase A2, reactive oxygen species and lipid peroxidation in cerebral ischemia, Free Radic. Biol. Med. 40 (2006) 376–387.

[12] M. Santarius, C.H. Lee, R.A. Anderson, Supervised membrane swimming: small G-protein lifeguards regulate PIPK signalling and monitor intracellular PtdIns(4, 5) P2 pools, Biochem. J. 398 (2006) 1–13.

[13] L. Berti-Mattera, R. Peterson, M. Bell, J. Eichberg, Effect of hyperglycemia and its prevention by insulin treatment on the incorporation of 32P into polyphosphoinositides and other phospholipids in peripheral nerve of the streptozotocin diabetic rat, J. Neurochem. 45 (1985) 1692–1698.

[14] S. Jethmalani, A. Dang, F. Faas, Platelet phosphoinositide turnover in streptozotocin-induced diabetes, Prostaglandins Leukot. Essent. Fatty Acids 50 (1994) 339–346.

[15] S.A. Rajasekaran, S.P. Barwe, A.K. Rajasekaran, Multiple functions of Na, K-ATPase in epithelial cells, Semin. Nephrol. 25 (2005) 328–334.

[16] P. Xia, R.M. Kramer, G.L. King, Identification of the mechanism for the inhibition of Na+, K(+)-adenosine triphosphatase by hyperglycemia involving activation of protein kinase C and cytosolic phospholipase A2, J. Clin. Invest. 96 (1995) 733–740.

[17] R. Sampathkumar, M. Balasubramanyam, C. Tara, M. Rema, V. Mohan, Association of hypoglutathionemia with reduced Na^+/K^+ ATPase activity in type 2 diabetes and microangiopathy, Mol. Cell. Biochem. 282 (2006) 169–176.

[18] D. Raccah, C. Fabreguettes, J. Aulay, P. Vague, Erythrocyte Na+ K+ ATPase activity, metabolic control and neuropathy in IDDM patients, Diab. Care 19 (1996) 564–568.

[19] P. Baldini, S. Incerpi, S. Lambert-Gardini, A. Spinedi, P. Luly, Membrane lipid alterations and Na^+-pumping activity in erythrocytes from IDDM and NIDDM subjects, Diabetes 38 (1989) 825–831.

[20] P. Finotti, P. Palatini, Reduction of erythrocyte (NA+-K+)ATPase activity in type 1 (insulin-dependent) diabetic subjects and its activation by homologous plasma, Diabetologia 29 (1986) 623–628.

[21] D. Rahmani-Jourdheuil, Y. Mourayre, P. Vague, J. Boyer, I. Juhan-Vague, In vivo insulin effect of ATPase activities in erythrocyte membrane from insulin-dependent diabetics, Diabetes 36 (1987) 991–995.

[22] D.D. De La Tour, D. Raccah, M.F. Jannot, T. Coste, C. Rougerie, P. Vague, Erythrocyte Na/K ATPase activity and diabetes: relationship with C-peptide level, Diabetologia 41 (1998) 1080–1084.

[23] P. Vague, T.C. Coste, M.F. Jannot, D. Raccah, M. Tsimaratos, C-peptide, Na+, K(+)-ATPase, and diabetes, Exp. Diabesity Res. 5 (2004) 37–50.

[24] G. Ferretti, R. Rabini, T. Bacchetti, A. Vignini, E. Salvolini, F. Ravaglia, G. Curatola, L. Mazzanti, Glycated low density lipoproteins modify platelet properties: a compositional and functional study, J. Clin. Endocrinol. Metab. 87 (2002) 2180–2184.

[25] R. Wada, M. Sugo, M. Nakano, S. Yagihashi, Only limited effects of aminoguanidine treatment on peripheral nerve function, (Na^+, K^+)-ATPase activity and thrombomodulin expression in streptozotocin-induced diabetic rats, Diabetologia 42 (1999) 743–747.

[26] R. Wada, Y. Nishizawa, N. Yagihashi, M. Takeuchi, Y. Ishikawa, K. Yasumura, M. Nakano, S. Yagihashi, Effects of OPB-9195, antiglycation agent, on experimental diabetic neuropathy, Eur. J. Clin. Invest. 31 (2001) 513–520.

[27] M. Oldfield, L. Bach, J. Forbes, D. Nikolic-Paterson, E. McRobert, V. Thallas, R. Atkins, T. Osicka, G. Jerums, M. Cooper, Advanced glycation end products cause epithelial-myofibroblast transdifferentiation via the receptor for advanced glycation end products (RAGE), J. Clin. Invest. 108 (2001) 1853–1863.

[28] M.E. Westwood, O.K. Argirov, E.A. Abordo, P.J. Thornalley, Methylglyoxal-modified arginine residues—a signal for receptor-mediated endocytosis and degradation of proteins by monocytic THP-1 cells, Biochim. Biophys. Acta 1356 (1997) 84–94.

[29] R.N. Hull, W.R. Cherry, G.W. Weaver, The origin and characteristics of a pig kidney cell strain, LLC-PK1, In Vitro 12 (1976) 670–677.

[30] M.J. Ryan, G. Johnson, J. Kirk, S.M. Fuerstenberg, R.A. Zager, B. Torok-Storb, HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney, Kidney Int. 45 (1994) 48–57.

[31] Y.S. Mao, M. Yamaga, X. Zhu, Y. Wei, H.Q. Sun, J. Wang, M. Yun, Y. Wang, G. Di Paolo, M. Bennett, I. Mellman, C.S. Abrams, P. De Camilli, C.Y. Lu, H.L. Yin, Essential and unique roles of PIP5K-gamma and -alpha in Fcgamma receptor-mediated phagocytosis, J. Cell Biol. 184 (2009) 281–296.

[32] M. Mosior, D. Six, E. Dennis, Group IV cytosolic phospholipase A2 binds with high affinity and specificity to phosphatidylinositol 4, 5-bisphosphate resulting in dramatic increases in activity, J. Biol. Chem. 273 (1998) 2184–2191.

[33] J. Balsinde, M. Balboa, W. Li, J. Llopis, E. Dennis, Cellular regulation of cytosolic group IV phospholipase A_2 by phosphatidylinositol bisphosphate levels, J. Immunol. 164 (2000) 5398–5402.

[34] J. Casas, M. Gijon, A. Vigo, M. Crespo, J. Balsinde, M. Balboa, Phosphatidylinositol 4,5-bisphosphate anchors cytosolic group IVA phospholipase A2 to perinuclear membranes and decreases its calcium requirement for translocation in live cells, Mol. Biol. Cell 17 (2006) 155–162.

[35] E. Gabev, J. Kasianowicz, T. Abbott, S. McLaughlin, Binding of neomycin to phosphatidylinositol 4, 5-bisphosphate (PIP2), Biochim. Biophys. Acta 979 (1989) 105–112.

[36] K. Kotlo, S. Shukla, U. Tawar, R.A. Skidgel, R.S. Danziger, Aminopeptidase N reduces basolateral Na+-K+-ATPase in proximal tubule cells, Am. J. Physiol. Ren. Physiol. 293 (2007) F1047–F1053.

[37] D. Raccah, M. Lamotte-Jannot, T. Issautier, P. Vague, Effect of experimental diabetes on Na/K-ATPase activity in red blood cells, peripheral nerve and kidney, Diabete Metab. 20 (1994) 271–274.

[38] M. Tsimaratos, T. Coste, A. Djemli-Shipkolye, L. Daniel, F. Shipkolye, P. Vague, D. Raccah, Evidence of time-dependent changes in renal medullary Na, K-ATPase activity and expression in diabetic rats, Cell. Mol. Biol. 47 (2001) 239–245.

[39] D. Silverstein, M. Barac-Nieto, J. Falck, A. Spitzer, 20-HETE mediates the effect of parathyroid hormone and protein kinase C on renal phosphate transport, Prostaglandins Leukot. Essent. Fatty Acids 58 (1998) 203–213.

[40] A. Sanchez-Mendoza, P. Lopez-Sanchez, B. Vazquez-Cruz, A. Rios, S. Martinez-Ayala, B. Escalante, Angiotensin II modulates ion transport in rat proximal tubules through CYP metabolites, Biochem. Biophys. Res. Commun. 272 (2000) 423–430.

[41] T. Satoh, H. Cohen, A. Katz, Intracellular signaling in the regulation of renal Na+ K + ATPase. 1. Role of cyclic AMP and phospholipase A2, J. Clin. Invest. 89 (1992) 1496–1500.

[42] T. Satoh, H. Cohen, A. Katz, Intracellular signaling in the regulation of renal Na-K-ATPase. II Role of eicosanoids, J. Clin. Invest. 91 (1993) 409–415.

[43] L. Liu, K. Mohammadi, B. Aynafshar, H. Wang, D. Li, J. Liu, A.V. Ivanov, Z. Xie, A. Askari, Role of caveolae in signal-transducing function of cardiac Na+/K+-ATPase, Am. J. Physiol. Cell Physiol. 284 (2003) C1550–C1560.

[44] J. Liu, R. Kesiry, S.M. Periyasamy, D. Malhotra, Z. Xie, J.I. Shapiro, Ouabain induces endocytosis of plasmalemmal Na/K-ATPase in LLC-PK1 cells by a clathrin-dependent mechanism, Kidney Int. 66 (2004) 227–241.

[45] J. Liu, M. Liang, L. Liu, D. Malhotra, Z. Xie, J.I. Shapiro, Ouabain-induced endocytosis of the plasmalemmal Na/K-ATPase in LLC-PK1 cells requires caveolin-1, Kidney Int. 67 (2005) 1844–1854.

[46] M. Brizzi, P. Dentelli, R. Gambino, S. Cabodi, M. Cassader, A. Castelli, P. Defilippi, L. Pegorarro, G. Pagano, STAT5 activation induced by diabetic LDL depends on LDL glycation and occurs via src kinase activity, Diabetes 51 (2002) 3311–3317.

[47] S.J. Khundmiri, A.M. Bertorello, N.A. Delamere, E.D. Lederer, Clathrin-mediated endocytosis of Na+, K+-ATPase in response to parathyroid hormone requires ERK-dependent phosphorylation of Ser-11 within the alpha1-subunit, J. Biol. Chem. 279 (2004) 17418–17427.

[48] K.M. Weixel, R.S. Edinger, L. Kester, C.J. Guerriero, H. Wang, L. Fang, T.R. Kleyman, P.A. Welling, O.A. Weisz, J.P. Johnson, Phosphatidylinositol 4-phosphate 5-kinase reduces cell surface expression of the epithelial sodium channel (ENaC) in cultured collecting duct cells, J. Biol. Chem. 282 (2007) 36534–36542.

[49] D. Padron, Y.J. Wang, M. Yamamoto, H. Yin, M.G. Roth, Phosphatidylinositol phosphate 5-kinase Ibeta recruits AP-2 to the plasma membrane and regulates rates of constitutive endocytosis, J. Cell Biol. 162 (2003) 693–701.

[50] M. Krauss, M. Kinuta, M.R. Wenk, P. De Camilli, K. Takei, V. Haucke, ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma, J. Cell. Biol. 162 (2003) 113–124.

[51] A. Nakano-Kobayashi, M. Yamazaki, T. Unoki, T. Hongu, C. Murata, R. Taguchi, T. Katada, M.A. Frohman, T. Yokozeki, Y. Kanaho, Role of activation of PIP5K-gamma661 by AP-2 complex in synaptic vesicle endocytosis, EMBO J. 26 (2007) 1105–1116.

[52] J.R. Thieman, S.K. Mishra, K. Ling, B. Doray, R.A. Anderson, L.M. Traub, Clathrin regulates the association of PIPKIgamma661 with the AP-2 adaptor beta2 appendage, J. Biol. Chem. 284 (2009) 13924–13939.

[53] S.F. Bairstow, K. Ling, X. Su, A.J. Firestone, C. Carbonara, R.A. Anderson, Type Igamma661 phosphatidylinositol phosphate kinase directly interacts with AP2 and regulates endocytosis, J. Biol. Chem. 281 (2006) 20632–20642.

[54] T. Katori, C. Bannai, Y. Hayashi, K. Yamashita, Decreased Na, K-ATPase activity by glycation at the catalytic center, Horm. Metab. Res. 31 (1999) 5–7.

[55] A. Stevens, The contribution of glycation to cataract formation in diabetes, J. Am. Optom. Assoc. 69 (1998) 519–530.

[56] J. Imig, Eicosanoids and renal vascular function in diseases, Clin. Sci. 111 (2006) 21–34.

[57] B. Williams, R.W. Schrier, Glucose-induced protein kinase C activity regulates arachidonic acid release and eicosanoid production by cultured glomerular mesangial cells, J. Clin. Invest. 92 (1993) 2889–2896.

[58] M. Yorek, M. Stefani, S. Moore, Acute and chronic exposure of mouse cerebral microvessel endothelial cells to increased concentrations of glucose and galactose:effect of myoinositol metabolism, PGE2 synthesis and Na+/K+-ATPase transport activity, Metabolism 40 (1991) 347–358.

[59] S. Yamagishi, Y. Yamamoto, S. Harada, C. Hsu, H. Yamamoto, Advanced glycosylation end products stimulate the growth but inhibit the prostacyclin-producing ability of endothelial cells through interactions with their receptors, FEBS Lett. 384 (1996) 103–106.

[60] S. Yamagishi, Y. Inagaki, T. Okamoto, S. Amano, K. Koga, M. Takeuchi, Advanced glycation end products inhibit *de novo* protein synthesis and induce TGF-beta overexpression in proximal tubule cells, Kidney Int. 63 (2003) 464–473.

[61] N. Shanmugam, Y. Kim, L. Lanting, R. Natarajan, Regulation of cyclooxygenase-2 expression in monocytes by ligation of the receptor for advanced glycation end products, J. Biol. Chem. 278 (2003) 34834–34844.

[62] N. Shanmugam, I. Gaw-Gonzalo, R. Natarajan, Molecular mechanisms of high glucose-induced COX-2 regulation in monocytes, Diabetes 53 (2004) 795–802.
[63] F. Cipollone, A. Iezzi, M. Fazia, M. Zuccelli, B. Pini, C. Cuccurullo, D. De Cesare, G. De Blasis, R. Muraro, R. Bei, F. Chiarelli, A. Schmidt, F. Cuccurullo, A. Mezzetti, The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques, Circulation 108 (2003) 1070–1077.
[64] S. Owada, O. Larsson, P. Arkhammar, A. Katz, A. Chibalin, P. Berggren, A. Bertorello, Glucose decreases Na+ K+ ATPase activity in pancreatic beta-cells. An effect mediated via Ca2+-independent phospholipase A2 and protein kinase C-dependent phosphorylation of the alpha-subunit, J. Biol. Chem. 274 (1999) 2000–2008.
[65] A. Aperia, A. Bertorello, I. Seri, Dopamine causes inhibition of Na + -K + -ATPase activity in rat proximal convoluted tubule segments, Am. J. Physiol. (Renal Fluid Electrolyte Physiol) 252 (1987) F39–F45.
[66] J. Garvin, K. Sanders, Endothelin inhibits fluid and bicarbonate transport in part by reducing Na+/K+ ATPase activity in the rat proximal straight tubule, J. Am. Soc. Nephrol. 1991 (1991) 976–982.
[67] M. Zeidel, H. Brady, B. Kone, S. Gullans, B. Brenner, Endothelin, a peptide inhibitor of Na+, K+-ATPase in intact renal tubular epithelial cells, Am. J. Physiol. 257 (1989) C1101–C1107.
[68] I. Gaidarov, J.H. Keen, Phosphoinositide-AP-2 interactions required for targeting to plasma membrane clathrin-coated pits, J. Cell Biol. 146 (1999) 755–764.
[69] G. Rohde, D. Wenzel, V. Haucke, A phosphatidylinositol (4, 5)-bisphosphate binding site within mu2-adaptin regulates clathrin-mediated endocytosis, J. Cell Biol. 158 (2002) 209–214.
[70] M. Jost, F. Simpson, J.M. Kavran, M.A. Lemmon, S.L. Schmid, Phosphatidylinositol-4, 5-bisphosphate is required for endocytic coated vesicle formation, Curr. Biol. 8 (1998) 1399–1402.
[71] M.A. West, N.A. Bright, M.S. Robinson, The role of ADP-ribosylation factor and phospholipase D in adaptor recruitment, J. Cell Biol. 138 (1997) 1239–1254.
[72] S. Honing, D. Ricotta, M. Krauss, K. Spate, B. Spolaore, A. Motley, M. Robinson, C. Robinson, V. Haucke, D.J. Owen, Phosphatidylinositol-(4, 5)-bisphosphate regulates sorting signal recognition by the clathrin-associated adaptor complex AP2, Mol. Cell 18 (2005) 519–531.
[73] R. Zoncu, R.M. Perera, R. Sebastian, F. Nakatsu, H. Chen, T. Balla, G. Ayala, D. Toomre, P.V. De Camilli, Loss of endocytic clathrin-coated pits upon acute depletion of phosphatidylinositol 4, 5-bisphosphate, Proc. Natl Acad. Sci. USA 104 (2007) 3793–3798.
[74] Y. Kanaho, A. Nakano-Kobayashi, T. Yokozeki, Novel activation mechanism and physiological function of PIP5Kgamma661, Adv. Enzyme Regul. 48 (2008) 88–96.
[75] M. Krauss, V. Kukhtina, A. Pechstein, V. Haucke, Stimulation of phosphatidylinositol kinase type I-mediated phosphatidylinositol (4, 5)-bisphosphate synthesis by AP-2mu-cargo complexes, Proc. Natl Acad. Sci. USA 103 (2006) 11934–11939.
[76] G. Di Paolo, L. Pellegrini, K. Letinic, G. Cestra, R. Zoncu, S. Voronov, S. Chang, J. Guo, M.R. Wenk, P. De Camilli, Recruitment and regulation of phosphatidylinositol phosphate kinase type 1 gamma by the FERM domain of talin, Nature 420 (2002) 85–89.
[77] M.A. Barbieri, C.M. Heath, E.M. Peters, A. Wells, J.N. Davis, P.D. Stahl, Phosphatidylinositol-4-phosphate 5-kinase-1beta is essential for epidermal growth factor receptor-mediated endocytosis, J. Biol. Chem. 276 (2001) 47212–47216.
[78] M.R. Wenk, L. Pellegrini, V.A. Klenchin, G. Di Paolo, S. Chang, L. Daniell, M. Arioka, T.F. Martin, P. De Camilli, PIP kinase Igamma is the major PI(4, 5)P(2) synthesizing enzyme at the synapse, Neuron 32 (2001) 79–88.
[79] J.R. Halstead, K. Jalink, N. Divecha, An emerging role for PtdIns(4, 5)P2-mediated signalling in human disease, Trends Pharmacol. Sci. 26 (2005) 654–660.
[80] A.J. Ungewickell, P.W. Majerus, Increased levels of plasma lysosomal enzymes in patients with Lowe syndrome, Proc. Natl Acad. Sci. USA 96 (1999) 13342–13344.
[81] T. Basturk, Y. Altuntas, A. Kurklu, L. Aydin, N. Eren, A. Unsal, Urinary N-acetyl B glucosaminidase as an earlier marker of diabetic nephropathy and influence of low-dose perindopril/indapamide combination, Ren. Fail. 28 (2006) 125–128.